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TITLE: Quantifying the Effects of Preventive Foods on the Metabolism of a Prostate Carcinogen in Humans and in Prostate Cells Grown in Culture

PRINCIPAL INVESTIGATOR: James S. Felton, Ph.D.

CONTRACTING ORGANIZATION: Lawrence Livermore National Laboratory
Livermore, California 94550

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6. AUTHOR(S)
James S. Felton, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Lawrence Livermore National Laboratory
Livermore, California 94550

E-Mail: Felton1@llnl.gov

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13. ABSTRACT (Maximum 200 Words)

We are investigating the effects of foods associated with reduced prostate cancer risk on a dietary carcinogen known to be associated with cooked meat and elevated cancer risk. Cooked muscle meats contain potent mutagens and carcinogens belonging to the heterocyclic amine class of compounds. One of these, PhIP, is a genotoxic carcinogen that has been shown to cause DNA damage in prostate tissue and prostate tumor formation in rats. We have developed a method to quantify urinary metabolites of PhIP in human volunteers that have been fed a meal of cooked chicken. Using this method, we have shown that PhIP metabolism may be affected by diet and lifestyle factors and that broccoli, soy, and tomatoes may influence the relative amounts of PhIP metabolite excretion. At the cellular level we are investigating the metabolism of PhIP in human prostate cancer cells and are investigating the relati0nship between DNA damage and gene expression. This research uses state-of-the-art analytical measurement methods to support conclusions about the role of diet and prostate cancer in humans. Although still preliminary, our results indicate that other components of the diet may have an effect on the metabolism of a commonly-occurring food carcinogen.

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Table of Contents

INTRODUCTION:

This study is designed to determine primary interventions that will prevent PhIP from causing prostate cancer. We are investigating the effects of foods associated with reduced prostate cancer risk on a dietary carcinogen known to be associated with cooked meat and elevated cancer risk. Cooked muscle meats, a prominent component of the Western diet, contain potent mutagens and carcinogens belonging to the heterocyclic amine class of compounds. One of these, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a genotoxic carcinogen, causing mutations in bacteria (Malfatti, 1995) and mammalian cells in culture (Thompson, 1998). There have been several animal studies linking PhIP exposure to DNA damage in prostate tissue or prostate tumor formation (Stuart, 2000, Shirai, 1997, Shirai, 1998). In humans, prostate tissue has been shown to activate PhIP and DNA adducts have been detected in the tissue after metabolic activation (Williams, 2000).

PhIP is naturally formed in meats during the cooking process, with the highest levels found in grilled or fried meats. There are measurable amounts of PhIP in numerous foods, and in very well-done meats, PhIP can be found at levels up to 400 ng per gram of meat (Sinha, 1995). The human intake of PhIP varies with food type and cooking conditions and is estimated to range from nanograms to tens of micrograms per day (Layton, 1995). We have developed a method to quantify urinary metabolites of PhIP in human volunteers that have been fed a meal of cooked chicken. This method allows us to understand PhIP metabolism in humans and to measure the effects of potentially chemopreventive foods. At the cellular level we are investigating the metabolism of PhIP in human prostate cancer cells as well as the effect of several of the putative active ingredients in the potentially chemopreventive foods.

Progress during Year 3:
TASK 1: Determine the stability of PhIP metabolism

A) Determine the stability of PhIP metabolism within an individual over time. Three healthy, normal, male volunteers have been recruited to participate in this phase of the study. The results of this study are described in a manuscript that we have submitted to Journal of Chromatography B. A copy of this manuscript is included in the Appendix. This manuscript also describes the PhIP metabolite profiles of the 12 volunteers that have participated in the study to date.

B) Determine the assay variability of the same urine sample.

This task began during the last 6 months of the first year and is now complete. We performed repeated analysis of one urine sample to determine the stability of the

metabolites over time (in urine frozen at -20°C) and the reproducibility of the LC/MS/MS method. Because of volume constraints no further assay of the sample analyzed in years 1 and 2 was possible. The results of assays of two different urine samples over the course of a year are presented in Table 1. These numbers represent the average peak area of 3 injections of the same extraction.

Sample variability continues to be an issue for the urine analysis. Variation exists in both repetitive injections of the same extraction (20-30%) and in repetitive extractions of the same sample (50-75%). We believe that the primary factor contributing to the variation is the complex urine matrix itself, and we are continually updating our sample preparation procedures to attempt to reduce the interference from the urine matrix.

Table 1. Assay variation for two urine samples. Numbers represent peak area. Each peak area is the average of three injections.

Sample	Date Analyzed	N^2-OH-PhIP-N^3-glucuronide	N^2-OH-PhIP-N^2-glucuronide	PhIP-N^2-glucuronide	4'-PhIP-sulfate
#1	20-Jul-01	4112284	70851303	9313549	3066886
	29-Nov-01	19114417	108347514	12532600	3289184
	15- Jul-02	11361614	175069458	22102453	20661745
#2	13-Jul-01	1301697	33431818	16513757	3787285
	06-Sep-02	8887714	18687637	10805732	37302174
	19-Nov-02	6438132	28182704	5664031	26487648

Recent attempts to minimize matrix effects include changing the HPLC columns after a set number of injections and decreasing the amount of sample injected. We have found that injecting more sample mass in an attempt to maximize metabolite peaks actually decreases sensitivity by adding more matrix. We have tried new solid phase extraction columns, as well as combinations of columns with only slight gains in extraction efficiency and peak shape.

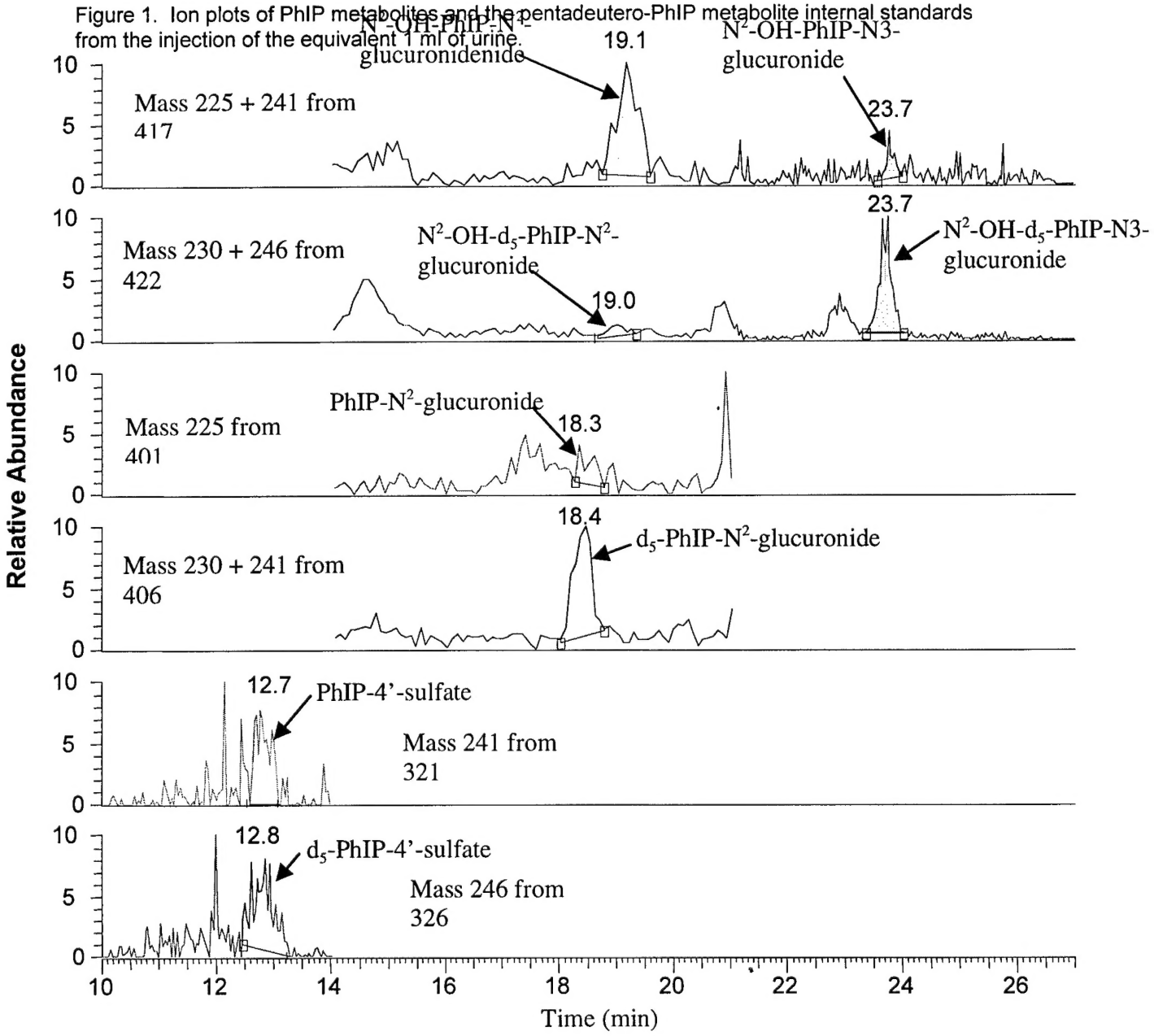
We have also changed the protocol to include the addition of deuterium-labeled internal standard for each of the metabolite peaks. This change, as well as other attempts to improve the analysis, is discussed in the manuscript included in the Appendix. At this time, we extract each urine sample a minimum of three times and inject each extraction three times in order to obtain reliable data. Figure 1 shows a chromatogram from a typical urine sample with the addition of the pentadeutero-PhIP metabolite internal standards.

We have found that the PhIP metabolites that we detect in the urine are stable stored at both –20 and 4°C. Analysis of urine samples up to 3 years old has shown little change in metabolite amounts.

TASK 2: Human Prostate Cells in culture
A) Effects of PhIP, N-OH-PhIP and 4'-OH-PhIP on cell proliferation

The effects of PhIP and a Phase I metabolism intermediate, N-OH-PhIP on cell growth in the prostate cancer cell lines LNCaP and PC3 was completed in Year 1. The effects of the other Phase I metabolism intermediate, 4'-OH-PhIP, have not been measured at this time. 4'-OH-PhIP is not available commercially and has proven to be more difficult to acquire than we expected. We now have received this compound from a collaborator, and will complete those tasks requiring 4'-OH-PhIP during the No Cost Time Extension (NCTE).

Figure 1. Ion plots of PhIP metabolites and the pentadeutero-PhIP metabolite internal standards from the injection of the equivalent 1 ml of urine.



Task 2, B) Macromolecular binding

This task was begun in Year 2 and will continue during the NCTE. To more definitively determine the DNA binding of PhIP in prostate cancer cells, the cells were treated with C14-labeled PhIP and NOH PhIP and were harvested for accelerator mass spectrometry (AMS). AMS is a technique that measures attamole levels of [14C]-labeled agents with high precision. Our group at Livermore has successfully been able to study xenobiotic metabolism and DNA binding using this technology (Turteltaub, 1990, Dingley, 1999).

In this experiment cells were dosed with 0, 0.003, 0.03, 0.3 and 3.0 µg/ml PhIP and NOH PhIP and harvested 4 hours later by trypsinization. Cells were homogenized and homogenates were snap frozen. DNA will be extracted for AMS analysis and mRNA will be extracted and analyzed for gene expression using a commercially available human gene microarray. Cells were also analyzed in parallel for the effect of these levels of PhIP on cell growth. When all of the data are collected, we will be able determine dose-dependent effects on DNA binding, gene expression and cell growth.

Task 2, C) Prostate cell metabolism

No further progress was made on this task during year 3. The metabolism of 4'OH PhIP will be investigated during the NCTE.

TASK 3: Link cellular metabolite profiles to urinary metabolite profiles

This task will be accomplished after more is known about the metabolites produced by the cells and we have more results from the macromolecular binding experiments. At this time, we have only found one of the PhIP metabolites in both the cells and the urine. This task will be accomplished during the NCTE.

TASK 4: Chemopreventive interventions

We have completed recruitment for the soy and broccoli interventions. At least four more men will be recruited to participate in the tomato intervention. Analysis of the urine samples will continue throughout the NCTE.

To investigate the effect of the intervention food on PhIP metabolism we quantify changes in PhIP urinary metabolites. In these studies, we fed the volunteers well-cooked chicken, collected urine and measured a baseline PhIP urinary metabolite profile. We then gave the subjects the intervention food daily for 3 days. On the fourth day we fed them chicken again and collected urine for another 24 hour period.

A) Effect of tomatoes on PhIP metabolism in humans and in prostate cells

We have recruited 4 volunteers to participate in this study to date. The intervention food for this study was 1/2 c. commercially available pasta sauce daily at lunch for three days.

To provide the human volunteers with a higher dose of lycopene that is still representative of a typical diet, we examined the literature to find the best food source. Cooked tomato products have the most lycopene. We analyzed three tomato products using a spectrophotometric assay published by Rao et al. in 1998 and Arias et al in 2000 (Rao, 1999, Arias, 2000). Three different samples of spaghetti sauce, Ragu Chunky Garden, Ragu traditional, and Prego Roasted Pepper were analyzed along with a negative control of a marinade sauce that contained no tomato products. These were extracted using hexane/acetone/methanol and the absorbance of the organic layer read in a spectrphotometer at 502 nm. All three tomato-containing sauces had lycopene, but not the marinade negative control. The Ragu Traditional sauce contained the most lycopene, about 30% more than the Ragu Chunky Garden and about five times more than the Prego Roasted Pepper. Thus the Ragu Chunky sauce was fed to the volunteers.

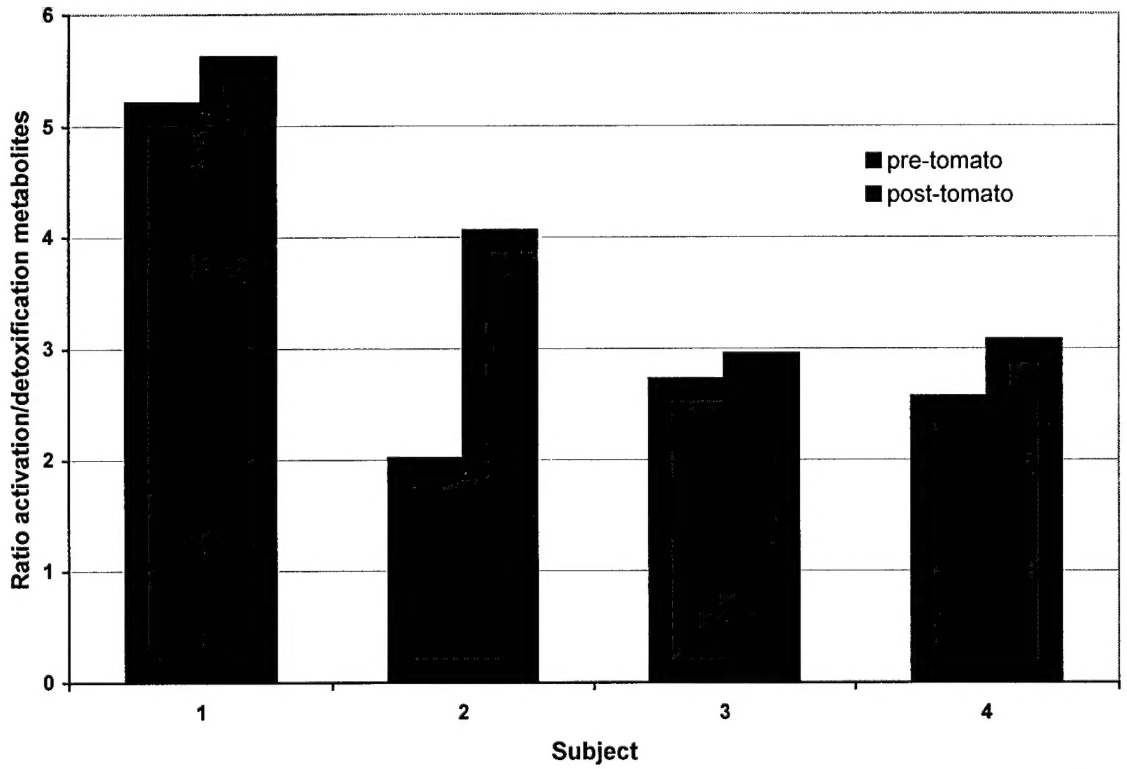


Figure 2. Effect of tomatoes on metabolite excretion. The ratio of the activation metabolites (N^2-OH-PhIP-N^2 glucuronide + N^2-OH-PhIP-N3-glucuronide) to the detoxification metabolites (PhIP-N^2-glucuronide+ 4'-PhIP-sulfate) is compared before and after the tomato intervention.

Preliminary analysis of the urine of the four men who participated in this study shows that there is a trend towards an increase in the activation metabolites (N^2-OH-PhIP-N^2 glucuronide and N^2-OH-PhIP-N3-glucuronide) compared to the detoxification metabolites (PhIP-N^2-glucuronide+ 4'-PhIP-sulfate) after the tomato intervention Figure 2. There is also a trend towards an increase in metabolite excretion in the 12-24 hour time period (Figure 3). Both of these results are similar to the effects that are seen with soy and are discussed further in section B. However, the changes that we see are very small, the population is limited and the urine analysis must be repeated, so these results should be considered preliminary.

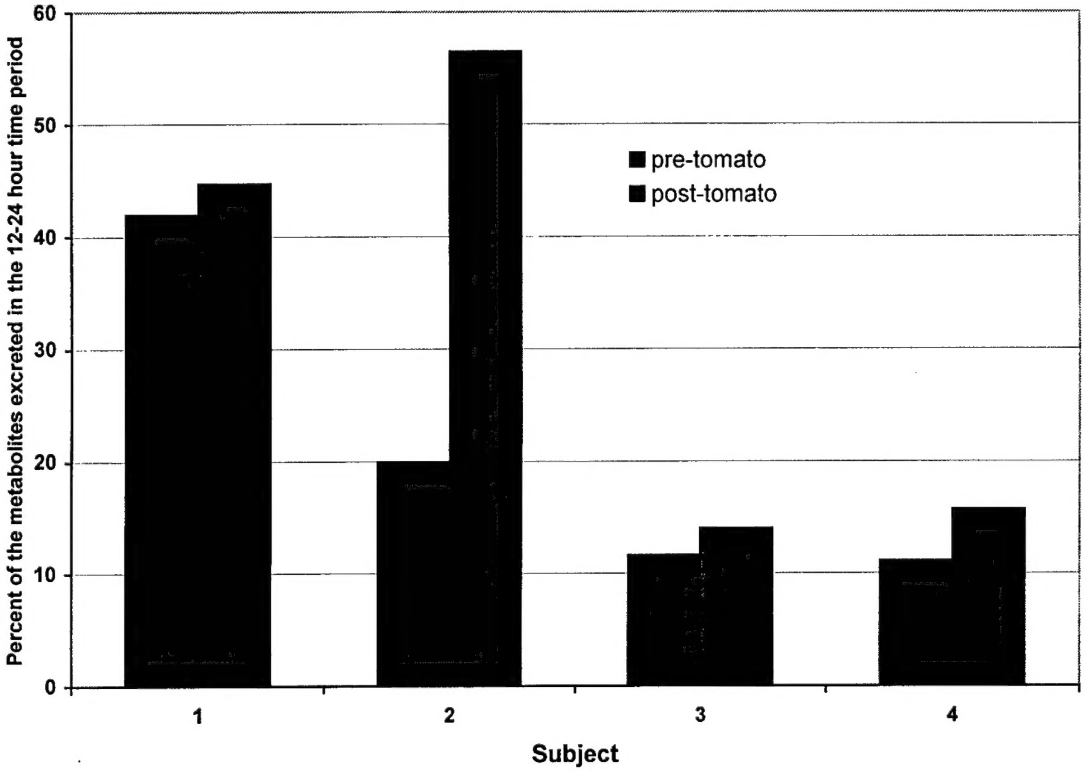


Figure 3. Effect of tomatoes on metabolite excretion rate. The percent of metabolites excreted in the 12-24 hour time period is compared before and after the tomato intervention.

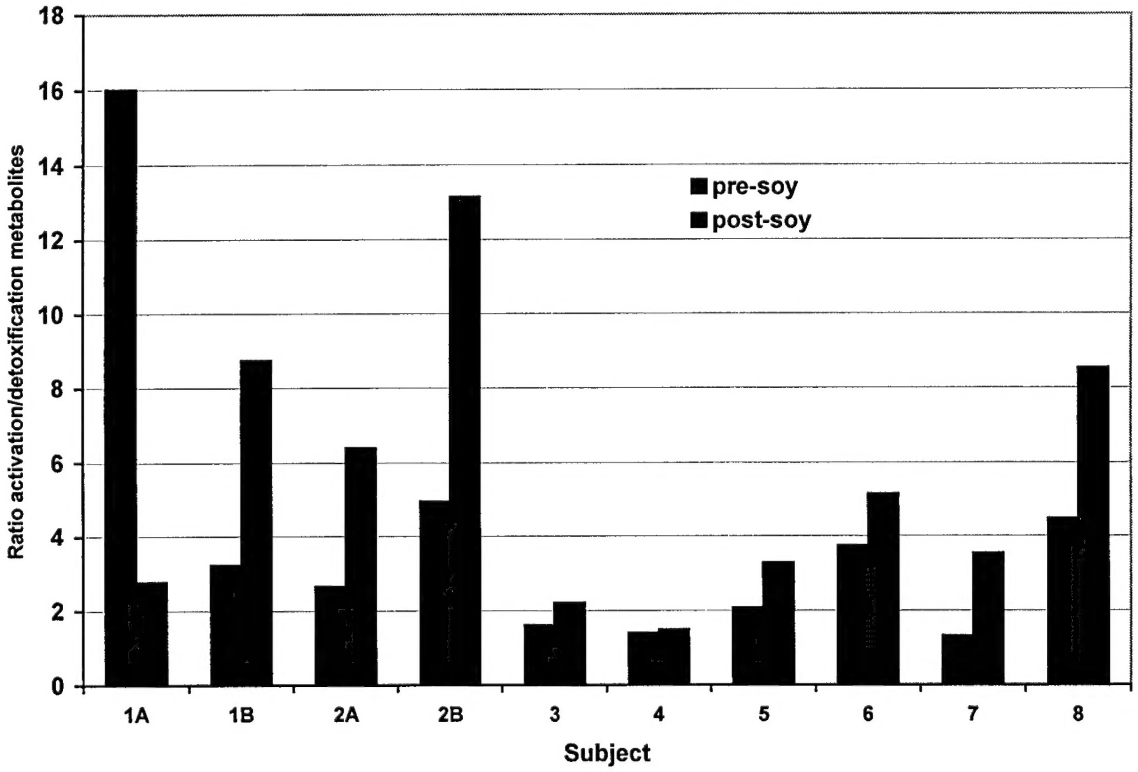
The effect of lycopene in cells will be accomplished in the NCTE. Lycopene is unstable in aqueous cell medium (half-life less than 2 hours), and it has been suggested that solubilization in micelles provides a more stable delivery system (Xu, 1999). We are currently working out the method for micellular formation.

B) Effect of soy on PhIP metabolism in humans and genistein in prostate cells

We have analyzed the urine from the seven volunteers who have participated in the soy intervention. In this trial the intervention food was a "soy shake" which contained 8 ounces of soy milk, 1 TBSP of a commercially available soy powder, bananas and honey. The shake was provided to the volunteers daily for 3 days. Although analysis of the samples is still on-going and data interpretation may change, it appears that there is a trend toward an increase in the ratio of the

activation metabolites (N^2-OH-PhIP-N^2 glucuronide and N^2-OH-PhIP-N3-glucuronide) compared to the detoxification metabolites (PhIP-N^2-glucuronide+ 4'-PhIP-sulfate). During Phase I metabolism PhIP is oxidized via cytochrome P4501A2 (CYP1A2) enzymes to a hydroxylated intermediate, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (*N*-hydroxy-PhIP). *N*-hydroxy-PhIP, which is itself mutagenic, can be converted to a biologically active form via Phase II metabolizing enzymes, primarily the acetyltransferases or sulfotransferases. This esterification generates electrophilic *O*-sulfonyl and *O*-acetyl esters, which have the capacity to bind DNA and cellular proteins (Buonarati, 1990, Edwards, 1994, Ozawa, 1994, Boobis, 1994). PhIP can also be hydroxylated at the 4 position, forming 2-amino-1-methyl-6-(4'-hydroxy) phenylimidazo[4,5-*b*]pyridine (4'-hydroxy-PhIP), which is not mutagenic. 4'-hydroxy-PhIP can be conjugated by sulfation and glucuronidation to polar compounds that are readily excreted (Buonarati, 1992, Watkins, 1991). In addition, the parent compound can be directly glucuronidated at the *N* ^{2} and *N*3 positions. These glucuronides are not reactive and this is believed to be a detoxification pathway (Kaderlik, 1994, Styczynski, 1993). Because *N*-hydroxy-PhIP is the first step of the activation pathway, we believe that the N^2-OH-PhIP-N^2-glucuronide and N^2-OH-PhIP-N3-glucuronide metabolites represent activation pathways metabolic products, whereas the PhIP-N^2-glucuronide and 4'-PhIP-sulfate represent detoxification pathways.

iFgure 4. Effect of soy on metabolite excretion. The ratio of the activation metabolites (N^2-OH-PhIP-N^2 glucuronide + N^2-OH-PhIP-N3-glucuronide) to the detoxification metabolites (PhIP-N^2-glucuronide+ 4'-PhIP-sulfate) is compared before and after soy intervention.



With the exception of the first trial of Subject 1, the ratio of the activation metabolites to detoxification metabolites increased in all of the subjects.

Soy milk and soy powder are complex mixtures that contain a variety of biologically active substances; it is possible that one or several of the components in this mixture induce P4501A1, the enzyme responsible for N-hydroxylation of PhIP. Several recent studies have investigated the affect of soy protein on CYP protein expression and activity with varying results. Three studies showed an increase in protein expression or activity in CYP27B1, P4502A, CYP3A and CYP2B1 in various rodent models (Kallay, 2002, Mezei, 2002, Ronis, 1999). Another study in rats showed a decrease in mammary CYP1A1 activity and mRNA expression (Rowlands, 2001). Another study of humans taking soy extract showed no inducibility of P4503A (Anderson, 2003).

Soy also seems to affect the rate of metabolite excretion. With the exception of Subject 1, all of the subjects excreted more metabolites in the 12-24 collection period after the soy intervention. The increase in metabolite excretion during the latter half of the collection period is driven primarily by an increased excretion of NOH metabolites (data not shown). It is possible that the induced activity of the P450 enyzmes demonstrated in Figure 4 also prolongs the metabolite excretion.

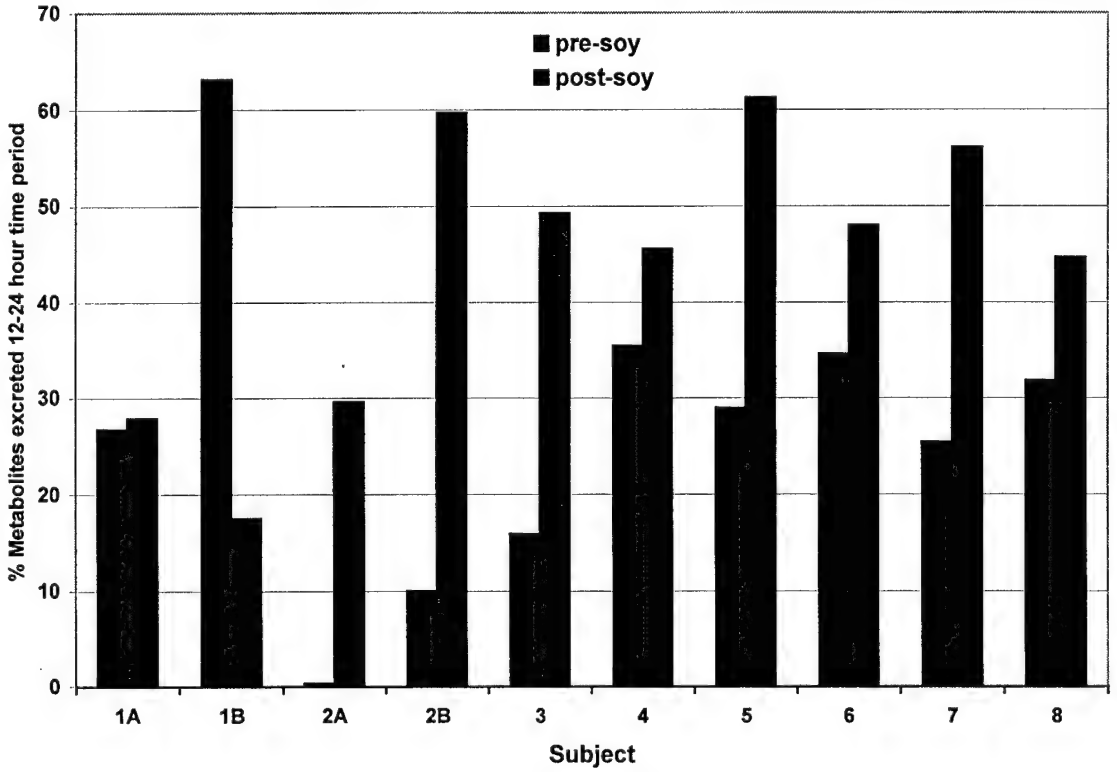


Figure 5. Excretion of PhIP metabolites in the 12-24 hour time period, before and after the soy intervention.

C) Effect of broccoli on PhIP metabolism in humans and sulforaphane in prostate cells

The progress on this task was discussed in the progress report for Year 1. No further work has been done on this task at this time.

KEY RESEARCH ACCOMPLISHMENTS:

- Determined that PhIP metabolite excretion is not stable over time and may be highly dependent upon diet and lifestyle factors.
- Determined that rate of excretion is the most constant factor in individuals over time
- Determined that approximately 25% of the PhIP dose measurable in the chicken can be detected in the urine.
- Individuals that excrete metabolites more quickly tend excrete more metabolites
- Determined that soy and tomato consumption may change the ratio of activation/detoxification metabolites excreted.

REPORTABLE OUTCOMES:

Manuscripts:

K.S. Kulp, M.G. Knize, N. Fowler, C.P. Salmon, and J.S. Felton, "PhIP metabolites in human urine after the consumption of well-cooked chicken". *Journal of Chromatography B,* submitted.

M.G. Knize, K.S. Kulp, C.P. Salmon, G.A. Keating and J.S. Felton, (2002) "Factors affecting the human heterocyclic amine intake and the metabolism of PhIP". *Mutation Research*, 506:153-162.

J.S. Felton, M.G. Knize, C.P. Salmon, M.A. Malfatti, and K.S. Kulp. (2002)"Human Exposure to Heterocyclic amine Food Mutagens/ Carcinogens: Relevance to Breast Cancer". *Environmental and Molecular Mutagenesis,* 39:112-118

Knize, M.G., Kulp, K.S., Malfatti, M.A., Salmon, C.P., and Felton, J.S. (2001)"An LC/MS/MS urine analysis method to determine human variation in carcinogen metabolism". *Journal of Chromatography A*, 914:95-103.

Posters and Presentations:

J.S. Felton, K.S. Kulp and M.G. Knize, "Mutagenic and carcinogenic chemicals in our diet". 4th International Conference of Environmental Mutagens in Human Populations, Florianopolis, SC, Brazil, May 4-8, 2003.

M.G. Knize, C.P. Salmon, K.S. Kulp, S.L. Fortson, and J.S. Felton, "Factors affecting heterocyclic amine intake". 2nd International Workshop on Analytical Chemical and Biological Relevance of Heterocyclic Aromatic Amines, Graz, Austria, May 7-9, 2003.

M.G. Knize, K.S. Kulp and J.S. Felton, "The effect of dietary soy protein on the metabolism of PhIP in humans" American Association of Cancer Research, Chemoprevention, Boston, MA, October, 2002

K.S. Kulp, M.G. Knize and J.S. Felton "Using human urinary PhIP metabolites to study individual variation in carcinogen metabolism and chemoprevention through dietary interactions" LLNL Science Day, September 2002

5/8/02 UC Davis Department of Engineering Seminar:
Technologies used in assessing risk from heterocyclic amines in cook food.

5/14/02 Sinai Medical and Cancer Center, NY, NY Seminar:
Human susceptibility to heterocyclic amines

9/24/02 Childrens Hospital Research Center, Oakland, CA Seminar:
Risk from heterocyclic amines in your diet

10/23/02 National Institutes of Health Staff Symposium on Diet and Health, Bethesda, MD,
Overcooking of Meat and the Impact on your health

11/15/02 University of California Toxic Substance Research and Teaching Program, UC Riverside Symposium
Human susceptibility to heterocyclic amines

11/19/02 National Toxicology Program/National Institute of Health, Bethesda, Md
Presentation at hearing on risk of heterocyclic amines

1/18/03 AACR Symposium on Molecular Epidemiology, Kona, HA

Mutagenicity in humans of cooked foods.

4/29/03 California State University, Hayward, Course on Diet and Cancer
Diet and Cancer

5/15/03 Impact of the Environment on Colon Cancer Symposium, Miami Beach, Florida
Mutagens in Food

Funding Applied for:

None during this reporting period.

CONCLUSIONS:

During the third year of the grant we submitted a manuscript detailing our conclusions about the stability of PhIP metabolism within an individual over time. We have also investigated the effect of soy and tomatoes on PhIP metabolism in humans. We have discovered that PhIP metabolism is affected by diet and lifestyle factors and may determine that soy and tomatoes affects PhIP metabolism.

We continue to have some problems with LC/MS/MS quantitation. This is not a trivial problem and has been reported to be an issue by many labs. We have changed some of the LC/MS and sample extraction procedures to improve sample variability.

This research uses state-of-the-art instrument measurement methods to support conclusions about the role of diet and prostate cancer in humans. Although still preliminary, our results indicate that other components of the diet, such as broccoli, soy, and tomatoes may have an effect on the metabolism of a commonly-occurring food carcinogen. Our investigations of the metabolism of PhIP and its intermediates and their effect on cellular response in prostate cancer cells may explain why this carcinogen specifically causes tumors of the prostate. It is possible that there are unique metabolic pathways present in prostate cells that produce a reactive intermediate that specifically causes DNA damage in the prostate.

We plan to finish the work proposed in the grant during the NCTE.

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APPENDICES:

K.S. Kulp, M.G. Knize, N. Fowler, C.P. Salmon, and J.S. Felton, "PhIP metabolites in human urine after the consumption of well-cooked chicken". *Journal of Chromatography B*

PhIP metabolites in human urine after consumption of well-cooked chicken

Kristen S. Kulp*, Mark G. Knize, Nicole Fowler, Cynthia P. Salmon, and James S. Felton

Biology and Biotechnology Research Program

P.O Box 808

Lawrence Livermore National Laboratory

Livermore CA, 94551-9900.

To whom correspondence should be addressed:

Kristen S. Kulp

Lawrence Livermore National Laboratory

Biology and Biotechnology Research Program

7000 East Ave., L-452

Livermore, CA 94550

Email:kulp2@llnl.gov

Phone: (925) 422-6351

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Abstract

We devised an assay to quantify the metabolites of PhIP in human urine following a single exposure of well-cooked meat. Four major human PhIP metabolites, N^2-OH-PhIP-N^2-glucuronide, PhIP-N^2-glucuronide, 4'-PhIP-sulfate, and N^2-OH-PhIP-N3-glucuronide, were found. N^2-OH-PhIP-N^2 glucuronide was the most abundant metabolite, although other metabolites varied among the population. The stability of PhIP metabolism over time was studied in three of the volunteers who repeated the assay 8 times over a 2.5 year period. PhIP metabolite excretion varied in each subject over time, although the rate of excretion was more constant. Our results suggest that quantifying PhIP metabolites should make studies of individual susceptibility and dietary interventions possible in the future.

Introduction

Potent genotoxic carcinogens of the heterocyclic amine (HA) class of compounds are produced in meat during cooking at high temperatures. The demonstrated mutagenicity of these compounds in bacteria [1], cells in culture [2,3] and mice [4], support the many studies of carcinogenicity in mice [5] and rats [6,7]. Mechanistic data show that, even at low doses, HAs form DNA adducts in rodents [8,9] and humans [10]. Of the 14 mutagens identified from cooked meat, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is the most mass abundant [11].

Humans are exposed to PhIP through the consumption of various cooked muscle meats, notably beef, pork and chicken [12-15]. The amount of PhIP that an individual is exposed to is related to food preparation methods [16-18], and the frequency of consumption. The presence of PhIP in restaurant and home-cooked meats has been documented, suggesting that humans may be exposed to PhIP in the range of 0.1 ng/g to over 200 ng/g by consuming these common foods [19-21]. These consumption levels may result in possible doses of micrograms per day for an individual.

The impact of heterocyclic amine exposure on human health is not clear, and its contribution to human cancer is a current subject of debate. Several epidemiology studies reported a positive correlation between the consumption of well-done meat and cancer risk [22-24]. In 1998, Zheng *et al.* described a

significant dose-dependence between meat preparation and breast cancer risk; women who preferred well-done hamburger, steak and bacon had a 4.6 fold greater risk of breast cancer than did women who preferred meats cooked "rare" or "medium" [25]. A recent case-control study of women in Shanghai, China showed a positive association of breast cancer risk and red-meat intake, especially well-done meat, which was more pronounced among women with a high body mass index [26]. Several studies reported an increased risk of colorectal adenomas and lung cancer with well-done and/or fried meat consumption [27-29]. African American males, who are at increased risk for prostate cancer, consume 2 to 3 times more PhIP than age-matched white males [30]. Two recent studies investigated the effect of N-acetyltransferase polymorphisms and cooked meat consumption on prostate cancer risk. Hein et al. found that a particular subset of NAT2 acetylator genotypes were at increased risk for prostate cancer [31]. In contrast, the study of Barrett et al. provided no support for the hypothesis that fast NAT2 acetylators are at increased risk of colon cancer, even if exposed to high levels of HAs from well-cooked meats [32]. Another study, performed in New Zealand, reported equivocal associations for well-done meat and prostate cancer [33]. Negative associations with cooked meat consumption have been reported with breast, colon, and rectal cancer [34-37].

PhIP is a procarcinogen that must be metabolically activated in order to damage DNA [38,39]. During Phase I metabolism PhIP is oxidized to the

hydroxlyated intermediates 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (N-OH-PhIP) or (2-amino-1-methyl-6-(4'-hydroxy) phenylimidazo[4,5-*b*]pyridine) 4'-OH-PhIP. Phase II metabolizing enzymes, primarily the acetyltransferases or sulfotransferases, then further convert N-OH-PhIP to a biologically active form that has been shown to bind DNA and cellular proteins [40-43]. Detoxification primarily involves glucuronidation. *N*-hydroxy-PhIP can form stable glucuronide conjugates at the N^2 and *N3* positions that can be excreted or transported to extrahepatic tissue for further metabolism [44,45]. 4'-hydroxy-PhIP can be conjugated by sulfation and glucuronidation to polar compounds that are readily excreted [46,47]. In addition, the parent compound can be directly glucuronidated at the N^2 and *N*3 positions. These glucuronides are not reactive and this is believed to be a detoxification pathway [45,48].

Human PhIP metabolism has been most intensively studied using hepatic microsomes or cells in culture. A recent study comparing PhIP metabolism in human and rat hepatocytes showed that the major human biotransformation pathway of PhIP was cytochrome P4501A2 (CYP1A2)-mediated N-oxidation followed by glucuronidation at N-2 and N3 positions of PhIP. In contrast, rat hepatocytes transformed PhIP to 4'-OH PhIP as the primary product. Glucuronide and sulfate conjugates of 4'-OH PhIP were detected in human hepatocytes, but as relatively minor products [49]. Extrahepatic metabolism of PhIP has been demonstrated in breast, prostate, and colon. Studies have shown that human mammary cells have the capacity to metabolize the parent

compound PhIP as well as the hydroxylated intermediates [50-52]. PhIP is glucuronidated by UGT1A1 in the human colon carcinoma cell line Caco2 [53] and human prostate cells have also been shown to metabolize PhIP [54]- [55, Kulp, personal observations]. The metabolic pathways and the metabolites produced during PhIP bioactivation in these target organs have not been fully determined.

Other studies of human PhIP metabolic pathways have been done in healthy volunteers by quantifying urinary metabolites. Pioneering work examined the relationship of urinary excretion of the unmetabolized parent compound and the dose received in well-done hamburgers [56,57]. PhIP and PhIP conjugates have been quantified in human urine using acid- or alkalai- hydrolyis. These investigations demonstrate PhIP bioavailability, time course of excretion and the correlation between meat consumption and urinary metabolites but do not give information about specific metabolic pathways [58-62]. Identification of human PhIP metabolites was determined in studies that investigated PhIP metabolism following administration of [^{14}C]-labelled PhIP to patients undergoing cancer surgery [63-65]. In these studies body fluids and tissues were examined using accelerator mass spectrometry to investigate PhIP metabolic pathways. In 2002, Stillwell et al correlated the excretion of N^2 –(β-1-glucosiduronyl)-2-hydroxyamino-1-methyl-6phenylimidazo[4,5-*b*]pyridine, measured as the deaminated product 2-OH PhIP, to Cyp1A2 and NAT2 activity in 66 healthy subjects [66].

There have been four major PhIP metabolites identified in human urine: N^2-OH-PhIP-N^2-glucuronide, PhIP-N^2-glucuronide, PhIP-4'-sulfate, and N^2-OH-PhIP-N3-glucuronide [64]. Recently, we described a solid-phase extraction LC/MS/MS method for quantifying these four metabolites in human urine, following a meal of well-cooked chicken. We applied this method to characterize PhIP metabolism in healthy individuals receiving a known dose of naturally-produced PhIP [67,68]. We have also extended that method to examine the interactions of potentially preventive foods [69]. In the current study we describe PhIP metabolism of 12 male volunteers, 3 of whom collected urine at 4 month intervals during a more than 2 year time span.

Material and Methods

Study Design

The study protocol was reviewed and approved by the Institutional Review Board for Human Research at Lawrence Livermore National Laboratory. Informed consent was obtained from each subject prior to beginning the study. The individuals participating were recruited from the local workforce, were all male, in good health, non-smokers, and of normal weight.

Meat Preparation and Controlled Dietary Period

Meat preparation conditions have been described previously [67]. Briefly, boneless, skinless chicken breasts were cut into approximately 2.5 cm pieces and fried in a non-stick coated pan, for 35-40 minutes. A representative chicken sample was removed for heterocyclic amine (HA) analysis. HA analysis was performed according to previously published methods [19]. The study subjects were provided with 150 g chicken with other non-meat foods and beverages. Total PhIP dose depended on the exact chicken cooking time and was different for each batch of chicken cooked. The PhIP content in the various batches ranged from 61-131 ppb, providing doses of 9.2-19.6 μg PhIP in 150 g of cooked chicken. The PhIP dose was known for each subject. Two of the subjects repeated the assay eight times over the course of 2.5 years. A third subject repeated the assay seven times in the same time frame.

Subjects were asked to abstain from meat consumption for 24 hours prior to eating the well-done chicken breast. There were no other dietary restrictions. Control urine was collected before eating the chicken and for 24 hours after in 6 hour increments. Samples were coded, the volume recorded and stored frozen at -20°C until analysis.

Extraction of PhIP metabolites

Urine samples (5 ml) were spiked with internal standard of urine (100μl) from a rat dosed with pentadeutero-PhIP [70] (1 mg/day) generating the 4 PhIP metabolites we detect in human urines. Samples were first applied to a pre-

conditioned 60 mg Strata™ X SPE column (Phenomonex, Torrance, CA). Metabolites were eluted with 5 ml methanol. The elution aliquot was evaporated to dryness under nitrogen and the metabolites were re-dissolved in 2.5 ml 0.01M HCl. Proteins and high molecular weight contaminants were removed by filtering the solution through a Centricon® YM-3 centrifugal filter (Millipore Corp., Bedford, MA). The filtrate was applied to a pre-conditioned benzenesulfonic acid column (SCX, 500 mg, Varian Sample Preparation Products, Harbor City, CA) and the column washed with 3 ml of 10% (v/v) methanol/ 0.01M HCl. The metabolites were eluted onto a coupled C18 column (Bakerbond spe®, 1000 mg, J.T. Baker, Phillipsburg NJ) with 60 ml of 0.05M ammonium acetate, pH 8. The C18 column was washed with 3 ml of 5% (v/v) methanol/H_2O and eluted from the C18 column with 5 ml of 60% (v/v) methanol/ H_2O. The metabolites were dried under nitrogen and 1 ml urine equivalent was injected into the LC/MS/MS in a volume of 20 µl.

Chromatography was done on an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) equipped with a YMC ODS-A column (3.0 x 250 mm). Metabolites were eluted at a flow rate of 200 µl/min using a mobile phase of A (water/methanol/acetic acid, 97:2:1) and 25% B (methanol/water/acetic acid, 95:4:1) with a linear gradient to 100% B at 20 min and held for 5 min.

Analytes were detected with a mass spectrometer (model LCQ, Finnigan, San Jose, CA) in the MS/MS positive ion mode using an electrospray interface. A

capillary temperature of 240°C, a source voltage of 4.5 kV, a sheath gas of 70 units and 5% auxillary gas were used. An ion trap injection time of 1000 ms and one microscan were used.

Alternating scans were used to isolate $[M+H]^+$ ions at mass 417, 401, and 321 for natural PhIP metabolites, and 422, 406, and 326, for the pentadeutero-labeled internal standard metabolites. Collision energy was 25%. Daughter ions were detected at appropriate masses: 241 [M+H-glucuronic acid]$^+$ and 225 [M^+H-glucuronic acid-OH]$^+$ from 417 for the N-hydroxy-N^2 and N3 glucuronide, respectively, 225 [M+H-glucuronic acid]$^+$ from 401 for the PhIP N2 glucuronide, 241 [$M+H-SO_3$]$^+$ from 321 for PhIP-4'-sulfate. Ion fragments detected for the deuterated internal standards were 5 mass units greater than the natural PhIP metabolite fragments.

Sample analysis and statistics

The overall recovery of the metabolites was determined by spiking each urine sample with known amounts of deteurium-labeled metabolites obtained from the rat urine. Final metabolite amounts were adjusted for losses based on the recovery of the internal standards. Each urine sample was analyzed at least twice. Total metabolite excreted in each time period was calculated by multiplying by the urine volume. Peak areas were converted to masses based on a response factor for PhIP and then normalized to percent of the original PhIP dose

consumedin the chicken. Excretion rate was calculated by summing each of the 4 metabolites and calculating the percent of the total metabolites that was excreted in each time period. Spearman rank-correlation tests were used to determine the association between the excretion level of the N^2-OH-PhIP-N^2-glucuronide excreted and the ingested dose of PhIP. Thirty-three data pairs were used in the analysis; data from all 12 subjects as well as all individual subject's repetitions of the assay. Subjects were divided into "fast" and "slow " excretion groups based on a comparison of the amount of metabolite excreted in the 0-6 and 6-12 hour time intervals. Subjects that excreted more metabolite in the 0-6 hour interval were considered "fast", those that excreted more in the 6-12 hour time interval were considered "slow". Average metabolites excreted by the two groups were compared using the Student's T-test.

Results

Human PhIP metabolite excretion after a meal of well-cooked chicken

Our method using LC/MS/MS detects peaks for the four identified human PhIP metabolites as well as four deuterated internal standard peaks in a single chromatographic run. Figure 1 shows a set of mass chromatograms for a typical sample of the equivalent of 1 ml of urine injected. For increased sensitivity, the data acquisition was made over three segments, isolating mass 321 for 14 min, masses 417, 401, and 422 for 7 min, and mass 417 only for the final 5.5 min. Since other ion peaks are often present in the chromatograms that are not one of

the four identified PhIP metabolites (Figure 1), expected peak retention times and peak widths are compared to reference samples to confirm the identity of the PhIP metabolites. N^2-OH-PhIP-N^2-glucuronide and its deuterium-labeled analog are detected as broader HPLC peaks that fragment into two daughter ions. The sum of these two peak areas is used for quantitation (Figure 1). The N^2-OH-PhIP-N3-glucuronide is separated in time from the N^2-OH-PhIP-N^2-glucuronide and fragments to mass 225 only (Figure 1). HPLC column lifetime is a problem with these samples. We slurry-pack our own columns with 10 μm particle size resin and replace the column after 24 injections to give the best results for routine samples.

Control urine samples were collected before the well-done chicken was consumed, during the period that the volunteers abstained from eating cooked meat. No PhIP metabolite peaks were seen in the control samples from the 12 individuals. Total urine excreted after chicken consumption was collected for 24 h in 6 h increments. Metabolite values shown are corrected for the total volume of urine. Figure 2 shows the percentage of the ingested PhIP dose recovered in the urine as PhIP metabolites for the 12 subjects. Recovered doses varied 9-fold despite the fact that all urine was collected and amounts were normalized to account for differences in PhIP dose. The total amounts of each of the four individual metabolites excreted during the 24 h collection period are also shown in Figure 2 as variably shaded regions of the bars. N^2-OH-PhIP-N^2 glucuronide is the most abundant urinary metabolite in all individuals, comprising 44 (Subject K)

to 80% (Subject F) of the total metabolite excreted. N^2-PhIP glucuronide is the second most abundant metabolites for 8 of the 12 volunteers and these 2 metabolites together account for 77- 95% of the total metabolite excretion for these individuals. In 3 individuals (B, L, and M) N^2-OH-PhIP-N3-glucuronide was the second most abundant metabolite and in Subject H PhIP-4'-sulfate was second most abundant, comprising almost 30% of the total metabolite excreted.

Figure 3 shows the rate of excretion of the PhIP metabolites in time periods of 0-6, 6-12, 12-18 and 18-24 hours. Subject L did not provide a sample for the 12-18h period. Subjects F and G provided sample for the 18-24 hour time period, but no metabolites were detected in these samples. In all of the subjects, the majority of the metabolites were excreted in the first 12 hours (61-92%). The individuals showed variation in the time of metabolite excretion. Six of the subjects (A, E, G, K, L and M) excreted more than 45% of the total metabolite in the 6-12 hour time period. The other six individuals excreted 34-50% of the total metabolite in the 0-6 h time period.

Correlation of metabolites excreted to PhIP dose ingested

A weak association was observed (Figure 4) between the amount of PhIP ingested and the total amount of N^2-OH-PhIP-N^2 glucuronide excreted in the 24 hour urine ($r_s = 0.29$, $p < 0.1$). Comparing the amount of PhIP ingested to the total metabolites excreted did not improve the correlation.

Comparing "fast" and "slow" excretion groups

The subjects in the study were divided into "fast" and "slow" excretion groups based on the amount of metabolites excreted in the 0-6h time period. Subjects were considered "fast" excretors if the ratio of the metabolites excreted in the 0-6h time interval to the 6-12h time interval was greater than or equal to 1. "Slow" excretors were defined as a ratio less than 1. The average metabolite excretion for each group is presented in Table 1. Subjects considered "fast" excreted significantly more N^2-OH-PhIP-N^2 glucuronide, 4'-PhIP sulfate and total metabolites than the subjects considered "slow" ($P<0.05$).

Human PhIP metabolism in 3 individuals over time

To determine changes in PhIP metabolism over time in individuals, we measured PhIP metabolite excretion in 3 subjects repeatedly over a 2.5 year time period (figures 5 and 6). The assay was repeated at approximately 4 month intervals. Subject C did not participate in the assay in December 1999. As seen in Figure 5, the amount of PhIP metabolites excreted, expressed as percent of the PhIP dose ingested, is not constant in these individuals over time. Considerable variation exists not only in the amount of each individual metabolite excreted (shown as variably shaded regions within the bar) but in the total amount of the PhIP dose excreted as well. In contrast, the rate of metabolite excreted is more constant (Figure 6). For Subject A, the larger fraction of the metabolites excreted was always in the later time intervals; 6-12 hours or 12-18 hours. Subject B, on the other hand, tends to excrete metabolites more quickly; in 5 of the 8 trials the

largest fraction of the metabolites were excreted in the 0-6 hour time interval. Subject C, similarly to Subject B, excreted the largest fraction of the metabolites in the 0-6 time interval in 4 out of 7 trials. Both B and C excreted almost all of the metabolites in first 12 hours after consuming chicken (an average of 80% for both subjects over all time intervals), whereas Subject A excreted an average of 64% of the metabolites in the first 12 hours.

Discussion

The opportunity to study a genotoxic dietary carcinogen at realistic levels in humans is rare. PhIP is of special interest because it causes tumors in animals and DNA damage *in vitro* in human tissues. The sites of PhIP damage in animal tumor studies are among the most common cancer sites in humans: the breast, colon, and prostate gland. In addition, exposure to PhIP need not be ubiquitous, but can be determined and modified through intervention, making PhIP exposure preventable.

The metabolism of PhIP has been well characterized in animals, however less is known about PhIP metabolism in humans. To take advantage of the opportunity to compare animals to humans, humans to each other, and see the influence of diet on carcinogen absorption and metabolism, we developed a method for quantifying PhIP metabolites in human urine. This study reports the variation in PhIP metabolism among twelve healthy human subjects.

Well-done chicken is the best source of PhIP exposure because at high temperatures and long cooking times chicken breast preferentially forms more PhIP and less of the related heterocyclic amines as compared to beef. This seems to be due to the higher amounts of the amino acids phenylalanine, isoleucine, leucine and tyrosine and lower amounts of glucose in chicken that favor the formation of PhIP [71]. Both the amounts of chicken consumed by our volunteers and the PhIP levels were comparable to consumption levels possible in households or restaurants [72].

In our study, the amount of metabolites excreted in the 0-24 hour urine represented 17 +/-10% of the ingested PhIP. In a previous study of normal females we reported a similar average of 21.5% of the PhIP dose recovered in the urine [67]. Strickland et al reported that 16.6% of the ingested PhIP could be quantified in the 0-12h acid-hydrolyzed urine of their population [60] and Stillwell et al reported the recovery of N-OH-PhIP-N^2-glucuronide (measured as 2-OH-PhIP) as an average of 24.6% [66]. These studies all confirm that PhIP present in the meat matrix is not completely bioavailable. In an earlier study of hospitalized elderly cancer patients given PhIP in a gelatin capsule, 90% of the ingested dose was recovered in the urine for two of the three subjects [64]. This indicates that PhIP provided in capsule form is more bioavailable than PhIP ingested in meat. We are currently investigating the bioaccessibility of PhIP from cooked meat using an *in vitro* digestion model. In that study we showed

that release of PhIP from the meat matrix was dependent upon pancreatic enzyme concentration and meat doneness [73]. We are also investigating the impact that other foods in the GI tract may have on PhIP bioaccessibility. Other factors such as transport across the intestinal cell monolayer and individual differences in metabolic pathway capacities may also ultimately affect how much of the ingested PhIP dose is recoverable in the urine.

The kinetics of PhIP metabolite excretion in our study are similar to those seen previously for humans [56,64,74]. Our results demonstrate that excretion times vary among the volunteers but that most of the dose is excreted in the first 18 hours. This suggests that these metabolites are suitable for investigating individual variation in rates and ratios of PhIP metabolism. Further, these metabolite measurements may be used as biomarkers of recent exposure, but are not suitable for long-term exposure estimates.

The detection of individual metabolites also confirms our earlier findings [64,74.] The major human PhIP metabolites are N^2-OH-PhIP-N^2-glucuronide, PhIP-N^2-glucuronide, PhIP-4'-sulfate, and N^2-OH-PhIP-N3-glucuronide. The ratio of the individual metabolites varied among our 12 individuals, although N^2-OH-PhIP-N^2-glucuronide was always the most abundant metabolite. In our previous study of female volunteers, we found that N^2-OH-PhIP-N^2-glucuronide was always the most abundant metabolite and PhIP-N^2-glucuronide consistently the second most abundant. In the current study, we found that N^2-OH-PhIP-N3-glucuronide and

PhIP-4'-sulfate, which were minor metabolites in the female population, contributed substantially to the total metabolite excretion. Other studies have investigated the effect of gender difference on PhIP dose-response relationships and excretion of 2-OH-PhIP and found no significant association [60,66]. However, neither of these studies identified and compared the excretion of the specific PhIP metabolites that are noticeably different in our studies. Further investigations in much larger populations will need to be done to confirm the trends that are present in our small population.

Our results demonstrate only a weak association between metabolites excreted and PhIP dose ingested. Other studies of more individuals have reported much stronger correlations [60,66]. It is possible that as we increase the number of subjects in our population, the correlation will become more robust.

Analyzing the average metabolite excretion of the "fast" versus "slow" individuals demonstrated that volunteers who excreted metabolites more quickly excreted significantly more N^2-OH-PhIP-N^2-glucuronide, PhIP-4'-sulfate and total metabolites than the individuals who excreted more slowly. It is possible that individuals that excrete more slowly excrete less metabolite because 1) less compound is being absorbed or it is being absorbed more slowly, 2) more of the compound is being sequestered in the tissues, or 3) the compound is being processed by other, unidentified pathways. However, due to the small size of the

study population, it is impossible to attribute meaning to these intriguing results. More work will need to be done in much larger populations to verify these trends.

We repeatedly analyzed PhIP metabolism in the same three individuals over time to determine the consistency of metabolite excretion. We found that both the percent of the dose excreted in the urine as well as the amounts of each of the metabolites was highly variable in each individual. Although the rate of metabolite excretion appeared to be more constant over time (one of the individuals was consistently slow, the other two were more often fast), the percentages of metabolites that were measured in each time interval also varied widely. Given the numerous reports of diet and lifestyle affecting metabolizing enzyme activity it is not surprising that there are metabolic variations in individuals eating a normal diet over time. Although these differences may make correlating PhIP metabolite excretion with genotype more difficult, it does suggest that it is possible to devise dietary intervention strategies to reduce the impact of PhIP exposure. Of the metabolites we detected, two appear to be part of the activation pathway for PhIP, N^2-OH-PhIP-N^2-glucuronide and N^2-OH-PhIP-N3-glucuronide [67]. It is likely that interventions that reduce the N-hydroxylation of PhIP or increase the direct glucuronidation of PhIP are desirable. We are currently investigating the effect of potentially chemopreventive foods on PhIP metabolism in small populations.

Altering the metabolism of PhIP to prevent formation of biologically active species may reduce individual susceptibility and prevent the occurrence of cancers in target tissues. . Our results suggest that quantifying PhIP metabolites should make studies of individual susceptibility and dietary interventions possible in the future.

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Table 1: Average Metabolite Excretion of "Fast" and "Slow" excretion groups

	N^2-OH-PhIP-N3-glucuronide	N^2-OH-PhIP-N^2-glucuronide	PhIP-N^2-glucuronide	PhIP-4'-sulfate	Total
Fast	3.2 +/- 3.2	20.6 +/- 15.1	5.5 +/- 5.1	2.5 +/- 1.9	31.8+/-19.6
Slow	1.6 +/- 1.4	10.4 +/- 6.9 [a]	2.7 +/- 1.9	0.9 +/- 0.6 [a]	15.6+/-8.2 [a]

Date are means +/- standard deviation. [a] = fast significantly different than slow, $p < 0.05$

Figure Legends:

Figure 1. Ion plots of PhIP metabolites and the pentadeutero-PhIP metabolite internal standards from the injection of the equivalent 1 ml of urine. See Materials and Methods for LC/MS/MS conditions.

Figure 2. Total 24h excretion of urinary PhIP metabolites for 12 subjects. Total excretion of each metabolite during the 24 hour time period was calculated and expressed as percent of the PhIP dose ingested.

N^2-OH-PhIP-N3-glucuronide N^2-OH-PhIP-N^2-glucuronide

PhIP-N^2-glucuronide PhIP-4'-sulfate

Figure 3. Rate of excretion of four PhIP metabolites for 12 subjects. Total urinary metabolites recovered during the 24 hours after dosing were quantified. Data represent the percentage of the total metabolites excreted during the designated time period. Time increments shown are: 0-6 hours 6-12 hours

12-18 hours 18-24 hours

Figure 4. Linear regression analysis of total N^2-OH-PhIP-N^2-glucuronide excreted 0-24 hours after chicken consumption as a function of the amount of the PhIP consumed for each individual.

Figure 5. Total 24h excretion of urinary PhIP metabolites for 3 subjects at several different times. Total excretion of each metabolite during the 24 hour time period was calculated and expressed as percent of the PhIP dose ingested. A: Subject A B: Subject B C: Subject C

N^2-OH-PhIP-N3-glucuronide N^2-OH-PhIP-N^2-glucuronide

PhIP-N^2-glucuronide PhIP-4'-sulfate

Figure 6. Rate of excretion of four PhIP metabolites for 12 subjects. Total urinary metabolites recovered during the 24 hours after dosing were quantified. Data represent the percentage of the total metabolites excreted during the designated time period. Time increments shown are: 0-6 hours 6-12 hours

12-18 hours 18-24 hours

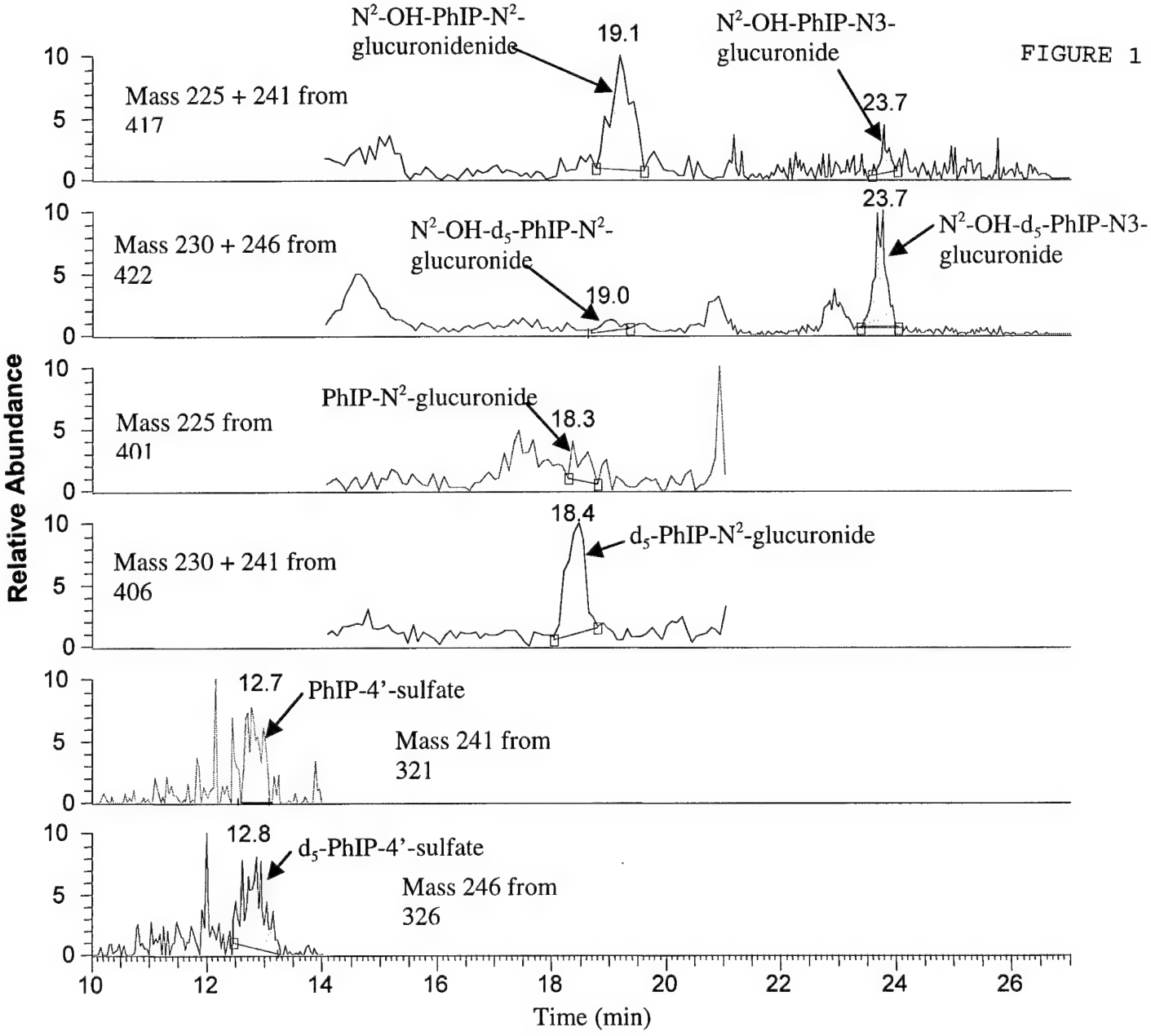

N2-OH-PhIP-N2-glucuronidenide
19.1
N2-OH-PhIP-N3-glucuronide
23.7
Mass 225 + 241 from 417
Mass 230 + 246 from 422
N2-OH-d5-PhIP-N2-glucuronide
19.0
23.7
N2-OH-d5-PhIP-N3-glucuronide
Mass 225 from 401
PhIP-N2-glucuronide
18.3
Mass 230 + 241 from 406
18.4
d5-PhIP-N2-glucuronide
12.7
PhIP-4'-sulfate
Mass 241 from 321
12.8
d5-PhIP-4'-sulfate
Mass 246 from 326
Relative Abundance
Time (min)

FIGURE 1

FIGURE 2

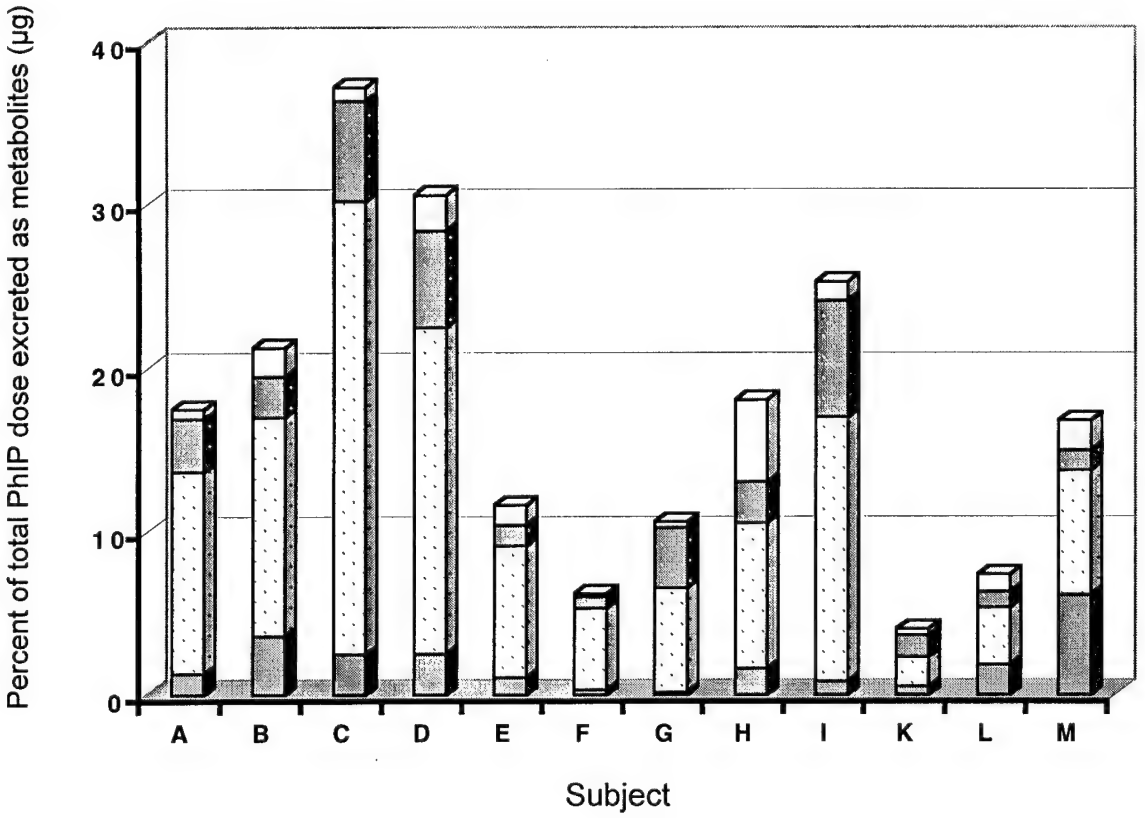

Percent of total PhIP dose excreted as metabolites (µg)
40
30
20
10
0
A
B
C
D
E
F
G
H
I
K
L
M
Subject

FIGURE 3

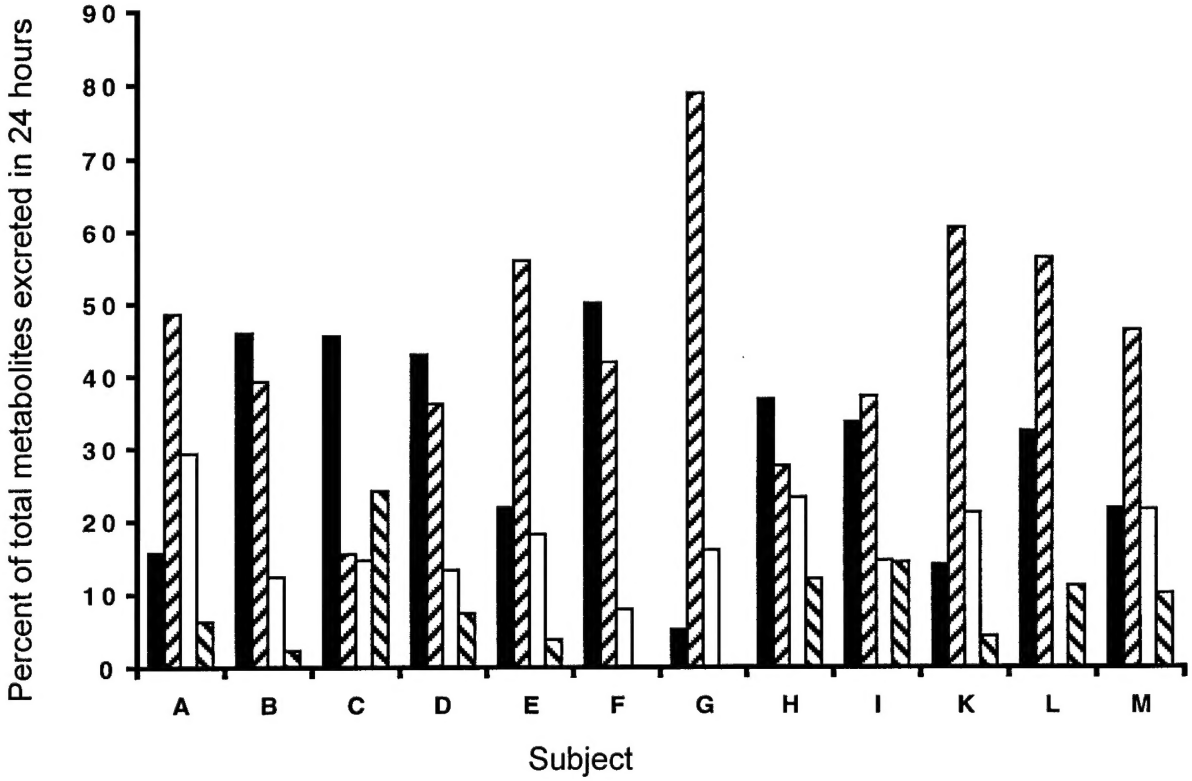

Percent of total metabolites excreted in 24 hours
90
80
70
60
50
40
30
20
10
0
A
B
C
D
E
F
G
H
I
K
L
M
Subject

FIGURE 4

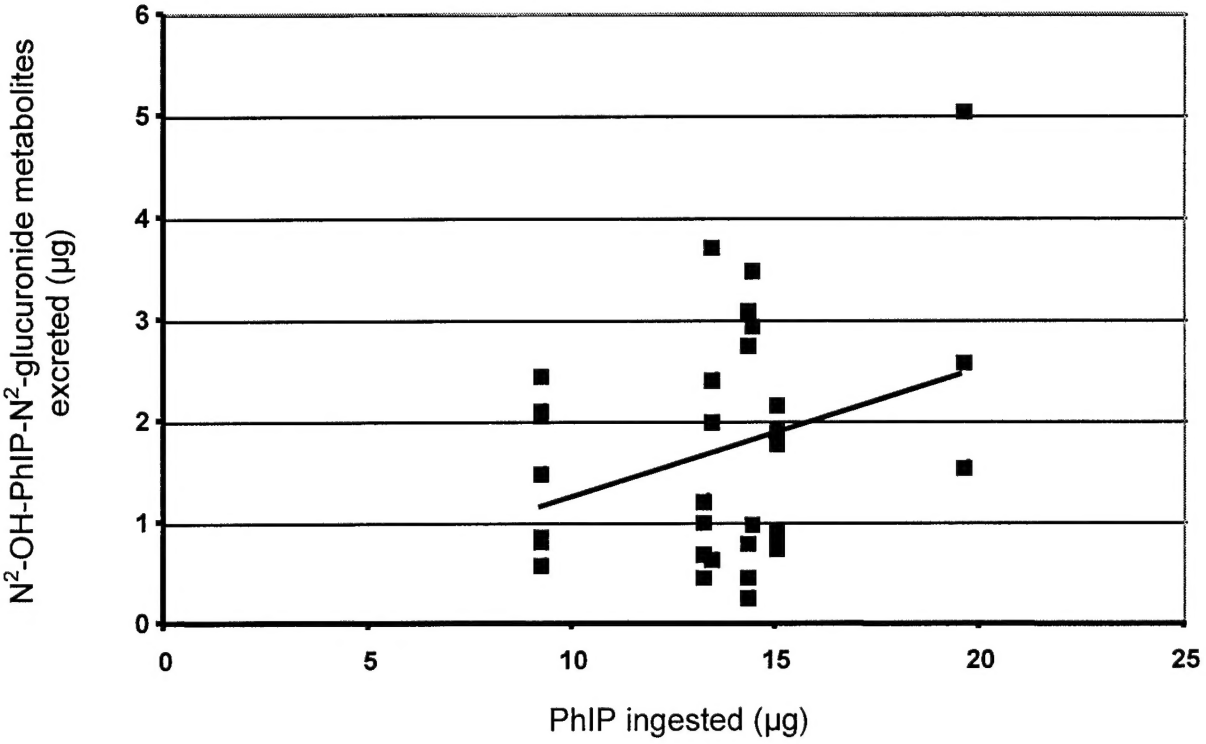

N2-OH-PhIP-N2-glucuronide metabolites excreted (µg)
0
1
2
3
4
5
6
0
5
10
15
20
25
PhIP ingested (µg)

FIGURE 5

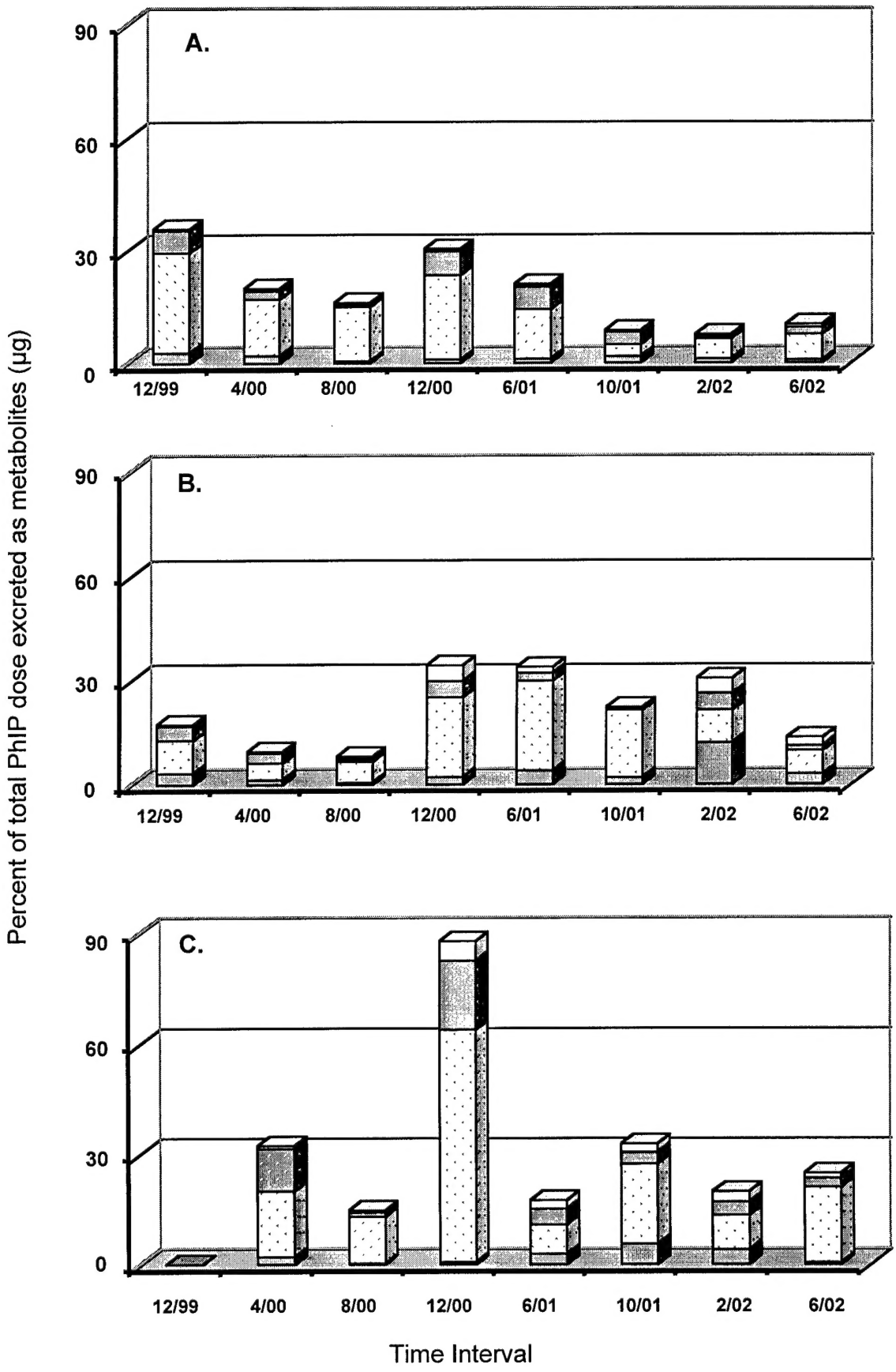
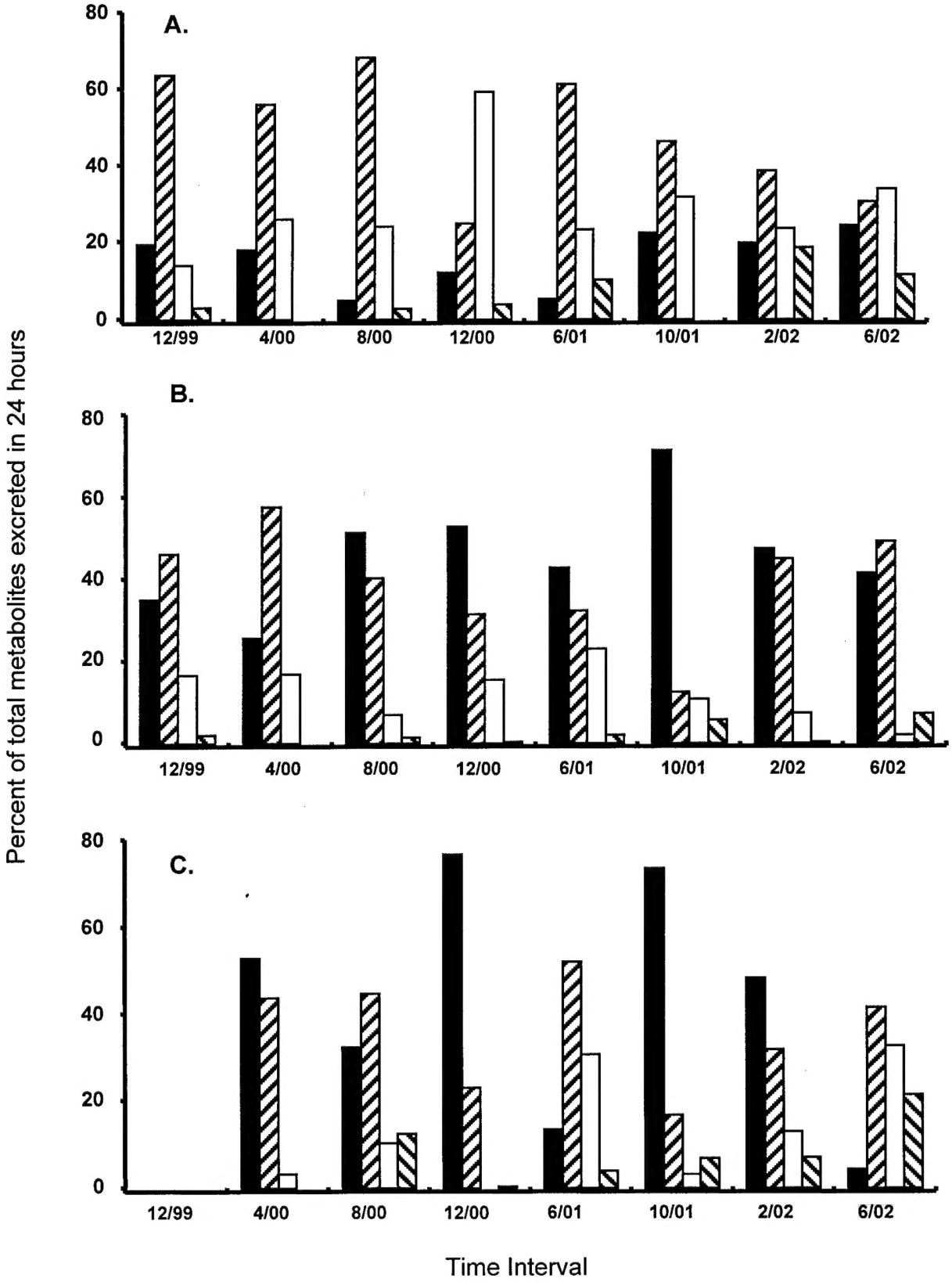

A.
B.
C.
90
60
30
0
12/99
4/00
8/00
12/00
6/01
10/01
2/02
6/02
Percent of total PhIP dose excreted as metabolites (µg)
Time Interval

FIGURE 6


A.
B.
C.
80
60
40
20
0
12/99
4/00
8/00
12/00
6/01
10/01
2/02
6/02
Percent of total metabolites excreted in 24 hours
Time Interval